Comprehensive Molecular Analyses of Lung Adenocarcinoma with Regard to the Epidermal Growth Factor Receptor, K-*ras*, MET, and Hepatocyte Growth Factor Status

Takamitsu Onitsuka, MD, Hidetaka Uramoto, PhD, MD,* Kenji Ono, PhD, MD,* Mitsuhiro Takenoyama, PhD, MD,* Takeshi Hanagiri, PhD, MD,* Tsunehiro Oyama, PhD, MD,† Hiroto Izumi, PhD, MD,‡ Kimitoshi Kohno, PhD, MD,‡ and Kosei Yasumoto, PhD, MD**

Background: The mutation and amplification of oncogenic genes are associated with carcinogenesis and tumor growth. The purpose of this study was to clarify the role of the epidermal growth factor receptor (EGFR), K-*ras*, MET, and hepatocyte growth factor (HGF) status in lung adenocarcinoma.

Methods: Tumor specimens were collected from 183 patients who underwent a complete resection for adenocarcinoma of the lung from 2003 to 2007 in our department. The genetic status of the EGFR and K-*ras* genes were investigated by polymerase chain reaction (PCR)-based analyses. Immunohistochemistry and real time PCR assays were used to evaluate the MET gene regarding to tyrosine phosphorylation and amplification, respectively. HGF status was evaluated by immunohistochemistry.

Results: The mutations of EGFR and K-*ras* were detected in 64 (35%) and 17 patients (9%), respectively. The tyrosine 1234/1235 phosphorylation of MET (p-MET 1234/1235) and MET amplification was identified in 12 (7%) and 8 (4%) specimens, respectively. Positive expression of HGF was identified in 104 specimens (57%). An EGFR mutation was found significantly more frequently in females and in tumors with wild type of K-*ras* and without MET amplification. A p-MET 1234/1235 was found significantly more frequently in the tumors with a positive expression of HGF. A multivariate survival analysis demonstrated that the wild type of K-*ras*, negative p-MET 1234/1235, and positive HGF expression were independently associated with an increased risk of poor survival.

Conclusions: The occurrence of MET amplification and EGFR/K-*ras* mutations might be mutually exclusive suggesting several distinct mechanisms in the development of lung adenocarcinoma. The wild type of K-*ras*, negative p-MET 1234/1235, and positive expression of HGF may be a useful marker for predicting poor prognosis of patients who underwent surgical resection of lung adenocarcinoma.

Key Words: EGFR, K-*ras*, MET, HGF, Lung cancer, Adenocarcinoma.

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Lung cancer is the leading cause of cancer-related deaths in Japan, and it kills more than 60,000 people per year.[1] The incidence of adenocarcinoma, one of the major histologic subtypes of non-small cell cancer (NSCLC), has been reported to be increasing recently.[2] It is a malignancy with a poor prognosis. Even in patients who achieve complete surgical resection, the 5-year survival is only around 50%.[3] Therefore, it is important to evaluate the malignant potential of tumor cells for a more precise evaluation of the prognosis of patients.

Somatically acquired mutations in the epidermal growth factor receptor (EGFR) gene in NSCLC are associated with a significant clinical response to EGFR-tyrosine kinase inhibitors (TKI).[4–7] However, most of the patients with this type of sensitive mutations in their tumor show acquired resistance during the TKI treatment.[8–11] The numerous findings for the resistant-related molecules lead to accumulation of the basic research. The p-MET initiates distinct signal transduction cascades involving such signaling molecules as Erk, Akt, and Stat3.[12] Deregulation of hepatocyte growth factor (HGF)-MET signaling may result in carcinogenesis in several solid tumors.[13] However, to our knowledge, few studies using cancer specimen have been conducted from a translational viewpoint. MET was identified as the receptor for HGF, and amplification of MET has been reported to be associated with 20% of lung adenocarcinoma with acquired resistance to EGFR-TKI.[14,15] Overexpression of HGF also may cause an acquired resistance to EGFR-TKI.[16] On the other hand, the K-*ras* mutation is associated with primary resistance to EGFR-TKI.[17]

*Second Department of Surgery, †Departments of Environmental Health, and ‡Molecular Biology, University of Occupational and Environmental Health, Kitakyushu, Japan.

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Address for correspondence: Hidetaka Uramoto, PhD, MD, Second Department of Surgery, School of Medicine, University of Occupational and Environmental Health, 1-1 Iseigaoka, Yahatanishi-ku, Kitakyushu 807-8555, Japan. E-mail: hidetaka@med.uoeh-u.ac.jp

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This is the first comprehensive molecular analysis of the EGFR, K-*ras*, MET, and HGF status with regard to the prognosis of patients with lung adenocarcinoma to elucidate their clinical significance.

PATIENTS AND METHODS

Patients and Clinical Features

Tumor samples were obtained from 296 patients with primary lung adenocarcinoma who had undergone a surgical resection between 2003 and 2007 in our department. Nine of these patients were stage IV and 25 underwent an incomplete resection. The tumor samples from 79 patients were too small to extract sufficient DNA for all of the analyses include the determination of the EGFR, K-*ras*, and MET amplification and to evaluate immunohistochemical (IHC) staining for p-MET 1234/1235 and HGF status. As a result, 113 patients were excluded from further analysis. Therefore, 183 tumor specimens were evaluated.

As genetic variables of lung adenocarcinoma, the EGFR mutations, K-*ras* mutation, p-MET 1234/1235, amplification of MET, and HGF expression were examined, and the correlation of these genetic factors with the clinicopathological variables were analyzed. The institutional review board's approved informed consent for the use of the tumor specimens was obtained either from all the patients or from the patient's legal guardians. The patients were followed-up every month within the first postoperative year and at approximately at 2 to 4 months intervals thereafter. The evaluations included a physical examination, chest roentgenography, an analysis of blood chemistry, and measurements of tumor markers. Chest and abdominal computed tomography, brain magnetic resonance imaging, and a bone scintiscan were performed every 6 months for 3 years after surgery. Additional examinations were performed if any symptoms or signs of recurrence were detected. Twenty-seven (14.8%) patients had received adjuvant chemotherapy as follows: 18, carboplatin plus paxclitaxel; 7, carboplatin plus gemcitabine; and 2, tegafur-uracil. Twelve patients received 250 mg/d of gefitinib for their recurrence. The median follow-up period was 34. 2 months, ranged from 1.0 to 70.0 months.

Detection of EGFR Mutations

The genomic DNA was extracted from each tumor. The EGFR mutations were examined by previously described methods, as reported previously.[19] Briefly, the exon 19 deletion of EGFR was detected by a simple screening method, which was the detection of shorter band than 147 base pair (bp) on agarose gel electrophoresis of the PCR product, derived from wild-type allele. The exon 21 L858R point mutation was detected by mutant-allele specific amplification. The 3′ ends of 22-bp oligonucleotides used as PCR primers corresponded to G for T of EGFR codon 858. That is, the sense-primer sequence for wild type was 5′-TCAAGATCACAGATTTTGGGCT, and that for L858R mutation was 5′-TCAAGATCACAGATTTTGGGCG. The antisense primer for both wild type and mutant type was 5′-CATCCTCCCCTGCATGTGTTAAAC. The PCR products were run on electrophoresis on a 2% agarose gel.

Detection of *K-ras* Mutations

A designed-restriction fragment length polymorphism method was used to detect K-*ras* codon 12 mutations.[19] Briefly, a sense-mismatched primer was used to introduce a new restriction site into the PCR product derived from the wild-type allele. The newly introduced restriction site was *Bst*NI for screening of codon 12. The wild-type alleles were digested and they yielded a smaller product (77 bp) than the mutant forms (97 bp), which were digestive resistant.

IHC Staining for Phosphorylation of MET

The 3-μm thick sections cut from paraffin-embedded specimens were prepared on glass slides for the phosphorylation of MET IHC staining. All of the specimens were stained with hematoxylin and eosin for the histopathologic diagnosis. The sections were placed in 0.01 mol/L citrate buffer (pH 6.0) and autoclaved at 121°C for 10 minutes. They were treated with 3% H_2O_2 for 5 minutes to block the endogenous peroxidase activity. The primary antibodies used were a monoclonal Ab against p-MET 1234/1235 (#3077, Cell Signaling Technologies, Danvers, MA), diluted 1:100 in phosphate buffered-saline, and incubated for 18 hours at 4°C. Thereafter, IHC staining was performed by the labeled polymer method (Histofine Simple Stain MAX-PO kit, Nichirei, Tokyo, Japan) according to the manufacturer's instructions. The positive and the negative controls were processed by the breast cancer specimen harboring phosphorylation of MET and the exclusion of the primary antibody, respectively. The stained specimens were then categorized into 8 degrees according to the IHC Allred score.[20] Initially, 6 degrees of the proportional score for the positive staining cells were assigned according to the frequency of positive tumor cells (0, none; 1, $<$1/100; 2, 1/100 to 1/10; 3, 1/10 to 1/3; 4, 1/3 to 2/3; and 5, $>$2/3). Thereafter, 4 degrees for the intensity score were assigned according to the intensity of the staining (0, none; 1, weak; 2, intermediate; and 3, strong). The proportional score and the intensity score were then added to each other to obtain a total score, which ranged from 0 to 8. According to the total IHC Allred score, the MET phosphorylation of the tumor was categorized as a negative expression when the score was 0 to 2 and a positive expression when the score was 3 to 8. The slides were independently examined by two of the investigators (T. O. and H. U.) who were blinded to the clinicopathological data. When a discrepancy was found between the two investigators, a consensus was reached via their simultaneous examination using a double-headed microscope.

Detection of *MET* Amplification

The *MET* gene copies were examined by previously described methods.[15] The *MET* gene copy number was analyzed by a quantitative real-time PCR method, performed on a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA) using a Fast SYBR Green Master Mix (Applied Biosystems). Each DNA sample of tumors was quantified by comparing the target locus to beta actin as an internal control. The quantification was based on the standard curves from a serial dilution of the human normal genomic DNA. PCR was performed for each primer set in triplicate,

and the mean value was calculated.[15] Amplification was defined as a copy number more than 1.31 copies, which was calculated by the mean of the *MET* gene copy number measured plus two times of standard deviation in this study.[21]

IHC Staining for HGF

The antibody used was a rabbit polyclonal anti-HGF Ab (sc-7949, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in phosphate buffered-saline, and incubated for 18 hours at 4°C.[15] The positive and the negative controls were processed by the normal gallbladder harboring over expression of HGF and the exclusion of the primary antibody, respectively. The proportion and intensity of stained cells were categorized into 8 degrees according to the IHC Allred score, and the results were categorized to positive and negative in the same manner as that for p-MET 1234/1235.

Statistical Analysis

Statistical associations were determined by the χ^2 test. The Cox proportional hazards model was applied to the multivariate survival analysis. The statistical difference was considered to be significant if the p value was less than 0.05. The data were analyzed with the use of Abacus Concepts, Survival Tools for Stat View (Abacus Concepts, Inc., CA).

RESULTS

Mutation of the EGFR and *K-ras*

All of the patients were Japanese. The subjects included 102 men and 81 women in this series, with a mean age of 68.5 years (range 23–88). The tumor stage was classified according to the Revisions in the International System for Staging Lung Cancer.[18] According to the pathologic stage, 113 patients were at stage IA, 35 at IB, 7 at IIA, 10 at IIB, 15 at IIIA, and 3 at IIIB. EGFR mutations were detected in 64 of 183 patients (35%). There were 41 of exon 21 L858R point mutation and 23 of exon 19 deletion mutation. The EGFR mutations were found significantly more frequently in females than males ($p < 0.001$). EGFR mutations, K-*ras* mutations, and MET amplification were all mutually exclusive. There were no patients with both EGFR and K-*ras* mutations, or EGFR mutations and MET amplification. No significant association of EGFR mutation was observed with age, pathologic stage, T status, N status, p-MET 1234/1235, or HGF expression. K-*ras* mutation was identified in 17 patients (9%). No significant association of K-*ras* mutation was identified with regard to various clinical factors, p-MET1234/1235, amplification of MET, and HGF expression (Table 1).

TABLE 1. Relationships Between Molecular Parameters and Clinicopathological Characteristics

			EGFR		K-*ras*		p-MET		MET Amplification		HGF Expression	
Variables	**Category**	**No. of Patients (n = 183)**	**Mut (%) = 64 (35)**	**Wt = 119**	**Mut (%) = 17 (9)**	**Wt = 166**	**P (%) = 12 (7)**	**N = 171**	**P (%) = 8 (4)**	**N = 175**	**P (%) = 104 (57)**	**N = 79**
Gender	Male	102	23 (23)	79	12 (12)	90	8 (8)	94	6 (6)	96	54 (53)	48
	Female	81	41 (51)	40[a]	5 (6)	76	4 (5)	77	2 (2)	79	50 (62)	31
Age (y)	<69	80	30 (38)	50	6 (8)	74	8 (10)	72	5 (6)	75	43 (54)	37
	≥69	103	34 (33)	69	11 (11)	92	4 (4)	99	3 (3)	100	61 (59)	42
Pathologic stage	I	148	54 (36)	94	12 (8)	136	9 (6)	139	5 (3)	143	80 (54)	68
	II–III	35	10 (29)	25	5 (14)	30	3 (9)	32	3 (9)	32	24 (69)	11
T status	T1	128	50 (39)	78	10 (8)	118	8 (6)	120	6 (5)	122	71 (55)	57
	T2–4	55	14 (25)	41	7 (13)	48	4 (7)	51	2 (4)	53	33 (60)	22
N status	Negative	152	54 (36)	98	13 (9)	139	10 (7)	142	5 (3)	147	83 (55)	69
	Positive	31	10 (32)	21	4 (13)	27	2 (6)	29	3 (10)	28	21 (68)	10
EGFR mutation	Mutated	64	—	—	0 (0)	64	3 (5)	61	0 (0)	64	37 (58)	27
	Wild	119	—	—	17 (14)	102[b]	9 (8)	110	8 (7)	111[c]	67 (56)	52
K-*ras* mutation	Mutated	17	0 (0)	17	—	—	2 (12)	15	0 (0)	17	13 (76)	4
	Wild	166	64 (39)	102[c]	—	—	10 (6)	156	8 (5)	158	91 (55)	75
p-MET	Positive	12	3 (25)	9	2 (17)	10	—	—	0 (0)	12	11 (92)	1
	Negative	171	61 (36)	110	15 (9)	156	—	—	8 (5)	163	93 (54)	78[d]
MET amplification	Positive	8	0 (0)	8	0 (0)	8	0 (0)	8	—	—	3 (38)	5
	Negative	175	64 (37)	111[d]	17 (10)	158	12 (7)	163	—	—	101 (58)	74
HGF expression	Positive	104	37 (36)	67	13 (13)	91	11 (11)	93	3 (3)	101	—	—
	Negative	79	27 (34)	52	4 (5)	75	1 (1)	78[d]	5 (6)	74	—	—

[a, b, c, d] Means statistically significant.
[a] $p < 0.001$.
[b] $p < 0.002$.
[c] $p < 0.034$.
[d] $p = 0.012$.
Mut, mutation; Wt, wild type; P, positive; N, negative; EGFR, epidermal growth factor receptor; p-MET, tyrosine 1234/1235 phosphorylation of MET; HGF, hepatocyte growth factor.

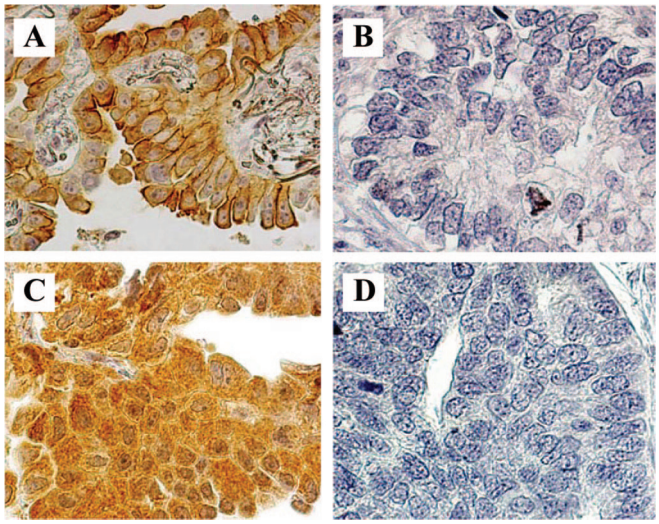

FIGURE 1. Representative immunohistochemical (IHC) staining. *A*, Positive expression of the p-MET 1234/1235 with brown stained membranes and cytoplasm is shown (original magnification 400×) the intensity score (IS) and proportional score (PS) were judged as 3 and 2, respectively. Therefore, IHC of tumor assumed a weak expression of 5. *B*, Negative expression of the tyrosine 1234/1235 phosphorylation of MET (p-MET 1234/1235) is shown (original magnification 400×). *C*, Positive expression of the hepatocyte growth factor (HGF) with brown stained cytoplasm is shown (original magnification 400×), the IS and PS were judged on 2 and 3, respectively. Therefore, IHC of tumor assumed a weak expression of 5. *D*, Negative expression of the HGF is shown (original magnification 400×).

IHC Analysis of p-MET 1234/1235

Staining of p-MET 1234/1235 was mainly found in the membranes and cytoplasm of cancer cells (Figure 1*A*). A positive p-MET 1234/1235 was identified in 12 patients (7%). A positive p-MET 1234/1235 was found more frequently in the tumors with positive expression of HGF ($p = 0.012$). No significant association of p-MET 1234/1235 was identified with the clinical factors and the amplification of MET (Table 1).

Analysis of *MET* Gene Amplification and IHC Analysis of HGF

MET amplification was identified only in eight patients (4%). No significant association of MET amplification was identified with the clinical factors and HGF expression. HGF staining was found mainly in the cytoplasm of cancer cells (Figure 1*C*). Positive HGF expression was identified in 104 patients (57%). No significant association of HGF expression was identified with the clinical factors (Table 1).

Prognosis of Patient According to the Molecular Parameters

The detectable relative risk is estimated 2.0 with 90% statistical power. A univariate analysis showed that old age, pathologic N status, negative p-MET 1234/1235, and positive expression of HGF were associated with an unfavorable prognosis (Table 2). A multivariate analysis demonstrated three variables (wild type of K-*ras*, negative p-MET 1234/1235, and positive expression of HGF) independently associated with the poor survival of patients (Table 3).

TABLE 2. Univariate Analysis Using a Proportional Hazard Model for the Survival of 183 Patients with Lung Cancer

		Univariate Analysis		
Variables	**Category**	**95% Confidence Interval**	**Hazard Ratio**	***p***
Gender	Male	0.880–11.582	1.181	0.269
	Female		1	
Age (yr)	<69	1.001–1.808	1.345	0.049
	≥69		1	
pT	T2–4	0.978–1.848	1.344	0.068
	T1		1	
pN	Positive	1.041–2.273	1.538	0.031
	Negative		1	
EGFR mutation	Positive	0.559–1.031	0.758	0.071
	Negative		1	
K-*ras* mutation	Positive	0.553–1.515	0.915	0.730
	Negative		1	
p-MET	Positive	0.236–0.826	0.441	0.011
	Negative		1	
MET amplification	Positive	0.461–1.942	0.947	0.881
	Negative		1	
HGF expression	Positive	1.004–1.828	1.355	0.047
	Negative		1	

EGFR, epidermal growth factor receptor; p-MET, tyrosine 1234/1235 phosphorylation of MET; HGF, hepatocyte growth factor.

TABLE 3. Multivariate Analyses of Various Prognostic Factors

	Characteristics				
Variables	**Unfavorable**	**Favorable**	**Risk Ratio**	**95% CI**	***p***
Gender	Male	Female	1.201	0.880–1.642	0.248
Age (yr)	≥69	<69	1.244	0.910–1.700	0.172
T status	2–4	1	1.161	0.834–1.618	0.376
N status	Positive	Negative	1.508	0.976–2.336	0.065
EGFR mutation	Negative	Positive	1.316	0.942–1.840	0.107
K-*ras* mutation	Negative	Positive	1.737	1.004–3.004	0.048
p-MET	Negative	Positive	2.865	1.484–5.525	0.002
MET amplification	Negative	Positive	2.004	0.851–4.717	0.112
HGF expression	Positive	Negative	1.506	1.095–2.070	0.012

95% CI, 95% confidence interval; EGFR, epidermal growth factor receptor; p-MET, tyrosine 1234/1235 phosphorylation of MET; HGF, hepatocyte growth factor.

DISCUSSION

An EGFR mutation was found limitedly to the tumors with wild type of K-*ras* and without amplification of MET. EGFR mutations, K-*ras* mutations, and MET amplification were all mutually exclusive. The MET amplification was observed in eight patients with wild-type EGFR and wild-type K-*ras*. These results are consistent with recent find-

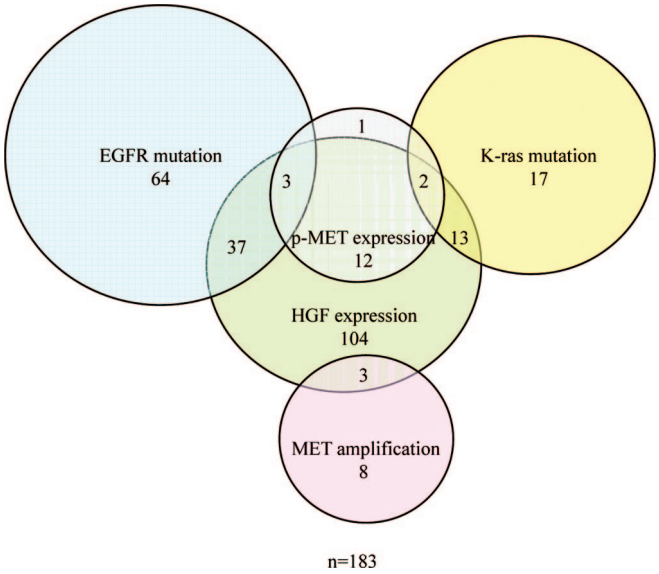

FIGURE 2. The Venn diagram illustrating the relationship among epidermal growth factor receptor (EGFR) mutation, K-*ras* mutation, MET amplification, and HGF overexpression in patients with lung adenocarcinoma ($n = 183$). The diameters of each circle are roughly proportional to the number of alterations. EGFR mutation, K-*ras* mutation, and MET amplification were mutually exclusive.

ings.[22–23] Onozato et al.[23] reported MET gene amplification mutually exclusive against mutation of EGFR, K-*ras*, and HER2. These findings suggest that distinct mechanisms may occur independently in tumors with EGFR, K-*ras*, and MET alterations during the process of development of lung adenocarcinoma (Figure 2).

The prevalence of positive p-MET 1234/1235, which is crucial in kinase activation,[24] was observed in 7% (12/183) of the tumors in this study. Nakamura et al.[25] reported that a tyrosine 1235 phosphorylation of MET using antityrosine 1235 phosphorylation Ab was positive in 22% (28 among 130 cases) of surgically resected Japanese lung adenocarcinoma. This discrepancy could be related to the difference of antibody, position of tyrosine-phosphorylation, and method used for IHC. Furthermore, tumors with positive p-MET 1234/1235 were found significantly more frequently in the tumors with positive expression of HGF ($p = 0.012$). The correlation between phosphorylation of MET and HGF expression supports the hypothesis that the HGF/MET loop may be activated in an autocrine fashion in cancer cells.[13] Negative p-MET 1234/1235 was significantly associated with poor survival in this study. These data seem to be inconsistent with those findings suggested that HGF/MET signaling is involved in cancer invasion.[26] However, Stella et al.[27] reported that activation of the Notch receptor resulted in transcriptional down-regulation of MET and impairment of HGF-dependent. In turn, MET activation leads to transcriptional induction of the Notch signaling indicating that MET is able to self-tune its own protein levels and the ensuring biochemical and biologic outputs through stimulating invasive growth of the Notch pathway. Nakamura et al.[25] reported that the tyrosine 1235 phosphorylation of MET had no influence on survival. Therefore, at present, the prognostic impact of tyrosine phosphorylation remains controversial, unless our results indicated the poor prognosis in patients with negative p-MET 1234/1235. On the other hand, over expression of HGF was significantly associated with poor survival, consistent with the results of previous studies.[28–30]

The amplification of MET was identified in only 8 of 183 patients (4%). This low rate of MET amplification is consistent with two recent findings,[23,31] which showed MET amplification in 5.6% (12 of 213) and 1.4% (2 of 148) in Japanese NSCLC. MET amplification was not associated with the clinical parameters, which is consistent with previous findings.[21]

Mutation of EGFR, mutation of K-*ras*, and amplification of MET seem to be mutually exclusive event in lung adenocarcinoma in this study (Figure 2). Both of EGFR mutations and MET amplification did not affect survival of patients in this study, which is consistent with the previous study.[32] The wild type of K-*ras* was associated with a poor survival ($p = 0.048$) in this study. In contrast, some reports have demonstrated that K-*ras* mutation may be associated with poor survival in NSCLC in white[33] and Japanese populations.[34,35] However, Graziano et al.[36] reported that K-*ras* mutation has a statistically significant correlation with poor survival only for the subgroup with stage II not with stage I NSCLC. Moreover, a recent study also showed that K-*ras* gene mutations are not independently associated with the poor prognosis for Japanese lung adenocarcinoma patients in whom 62% at stage I.[32] Therefore, the prognostic impact of K-*ras* gene mutation in lung adenocarcinoma remains controversial. These divergent results might be due to the difference of race, mutation frequency, stage of the disease, and statistical power related to number of patients examined.

The crosstalk between MET and EGFR, including the Akt or K-*ras* pathways, has been implicated in each signaling.[37] In the near future, molecularly targeted agent, such as EGFR-TKI for tumors with EGFR mutations, anti-EGFR monoclonal antibody (cetuximab) for lung adenocarcinomas without K-*ras* mutations, and MET inhibitor for those with MET amplification, and anti-HGF monoclonal antibody for those with HGF over expression, may bring benefits to decrease mortality and morbidity.[38,39]

In conclusion, these results indicate that the EGFR, K-*ras* mutation, and MET amplification are mutually exclusive suggesting presence of several distinct mechanisms in the development of lung adenocarcinoma. The wild type of K-*ras*, negative p-MET 1234/1235, and overexpression of HGF may therefore be useful markers for predicting poor prognosis in patients with resectable lung adenocarcinoma.

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